DE-1490

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In re Application of:	)	
	)	
Si-Hyoung LEE et al.	)	
	)	Group Art Unit: 1639
Serial No.: 10/613,855	)	
	)	Examiner: T. D. Wessendorf
Filed: July 3, 2003	)	

For: TRANSPOSON-MEDIATED RANDOM CODON-BASED MUTAGENESIS

Commissioner for Patents
P. O. Box 1450
Alexandria, VA 22313-1450

DECLARATION UNDER 37 C.F.R. §1.132

Sir:

I, Si-Hyoung LEE, being a citizen of the Republic of Korea and presently residing at Jugong 3-cha Apt. 308-701, Sinan-dong, Jinju-si, Gyungsangnam-do 660-769, Republic of Korea, do declare:

That I am one of the co-inventors of the invention disclosed in the above-identified application, and hence am fully familiar with the subject matter therein; and

That I have conducted an experiment as follows, in order to demonstrate that the method of the subject application is applicable to any DNA as a target. A DNA encoding phytase was used as a target DNA in the following experiment.

<Preparation of a substitution mutant library of phytase using the inventive random codon-based mutagenesis method>

(Step 1) Insertion and subsequent deletion of transposon at random sites of phytase gene

Phytase gene (*see* Exhibit A for its sequences) originating from *Bacillus licheniformis* SP4 strain was amplified by PCR using a forward primer, SP4-f (5'-CCGCTCGAGAGGAGGAAGGAAAATGAATCATTCAAAAACAC-3') and a reverse primer, SP4-r (5'-CGGACTAGTGTTTATTTTCCGCTTCTG-3. The amplified PCR product was purified with a PCR purification kit (Qiagen) and digested with *Xho* I and *Spe* I . The digestion product was eluted through 0.8 % agarose gel to obtain a DNA fragment of about 1.2-kb in size. The resulting DNA fragment was ligated to the *Xho* I /*Spe* I backbone of pBluescript II SK (Stratagene) to give a recombinant plasmid designated pBSK-SP4.

To insert Tn7-based transposon into plasmid pBSK-SP4, GPS-LS linker-scanning system (New England Biolabs) was employed. Transprimer-5 (New England Biolabs) was inserted into plasmid pBSK-SP4 according to the manufacturer's instructions, and *E. coli* MC1061 was transformed with the resulting plasmid. Positive transformants were selected by culturing them on LB-agar plates supplemented with 100 ㎍/㎖ of ampicillin and 15 ㎍/㎖ of kanamycin. The colonies (about 1 x 10^5) formed on the plates were collected and subjected to plasmid DNA extraction by Qiaprep Spin Miniprep (Qiagen). The extracted plasmid DNAs were treated with *Pme*I (New England Biolabs) which recognizes both termini of Tn7 to remove Tn7 and the resulting DNA was designated pBSK-

SP4-△Tn.

(Step 2) Removal of the nucleotides originating from the transposon and nucleotides of the phytase gene duplicated during the transposon insertion at one of the enzyme-digested ends of the phytase gene

In order to prepare a first cassette DNA to be inserted into the cut site of the phytase gene, a forward primer, phycas1-f (5'-GGGATCCTATGTATCCGCTCATGAGACAATAACC-3') and a reverse primer, phycas1-r (5'-GGGTCTGCACTCTTACACTAGATCCTTTTTGATC-3') were synthesized. PCR was conducted using pBC KS+ plasmid (Stratagene) as a DNA template and these primers. The PCR product was analyzed by 0.8 % agarose gel electrophoresis, and DNA fragments were extracted from the gel using a GENECLEAN kit (Bio 101).

The first cassette DNA thus purified was ligated to pBSK-SP4-△Tn prepared in Step 1 and *E. coli* MC1061 was transformed with the ligation product. The transformants were spread on LB-agar plates supplemented with 20 μg/mℓ of chloramphenicol. About 5 x 10^5 colonies grown on the plates were collected and subjected to plasmid DNA extraction by Qiaprep Spin Miniprep (Qiagen). The extracted plasmid DNA was digested with *Xho*I and *Spe*I. The DNA fragment carrying phytase gene in which the first cassette was introduced, was extracted. The extracted DNA was ligated to pBluescriptII SK plasmid digested with *Xho*I and *Spe*I, and *E. coli* MC1061 was transformed with the ligation product. The transformants were spread on LB-agar plates supplemented with 20 μg/mℓ of chloramphenicol. About 5 x 10^5 colonies grown on the plates were collected and subjected to plasmid

DNA extraction by Qiaprep Spin Miniprep (Qiagen). The extracted plasmid DNA was treated with *Bsg*I, and 3'-overhang were removed by T4 DNA polymerase (New England Biolabs). Specifically, 0.1～0.3 unit of T4 DNA polymerase was added per 1 μg DNA and reaction was carried out at 12℃ for 20 minutes. Then, 1 mM of EDTA was added to the reaction mixture and the resulting mixture was heated at 75℃ for 10 minutes to terminate the reaction. The resulting plasmid DNAs were treated with *Bam*HI and subjected to 1 % agarose gel electrophoresis. The DNA fragments, wherein the first cassette DNA was removed from the phytase gene, were extracted from the gel and designated pBSK-SP4-△CAS1.

(Step 3) Insertion of nucleotides, and removal of transposon-derived nucleotides and consecutive three nucleotides of the phytase gene from the other cut terminus of the phytase gene

In order to prepare a second cassette DNA to be inserted into the cut site of the DNA fragment obtained in Step 2, a forward primer, phycas2-f (5'-CGCGGATCCTATCTGCACTATGTATCCGCTCATGAGACAATAACC-3') and a reverse primer, phycas2-r (5'-NNNTTGACTCCTAGCCATTCTGCACTCTT CAVVTAGATCCTTTTTGA-3') having phosphorylated 5'-ends were synthesized. PCR was conducted using plasmid pBC KS+ as a DNA template and these primers. The second cassette DNA thus prepared was treated with *Bam*HI and ligated with pBSK-SP4-△CAS1 prepared in Step 2 using T4 DNA ligase. *E. coli* MC1061 was transformed with the ligation product, and the transformants were spread on LB-agar plates supplemented with 20 μg/mℓ of chloramphenicol. About 5 x 10^5 colonies grown on the plates were collected and subjected to plasmid DNA extraction by

Qiaprep Spin Miniprep (Qiagen). The extracted plasmid DNAs were treated with *Bsg*I.

The resulting DNA fragment was treated with T4 DNA polymerase to convert its ends into blunt ends.

(Step 4) Preparation of a mutant library by self-ligation of the target DNA

The DNA fragment obtained in Step 3 was subjected to self-ligation. *E. coli* MC1061 was transformed with the ligation mixture, and about 5 x 10^5 transformants were obtained and subjected to plasmid DNA extraction. The extracted plasmid DNAs were treated with *Xba*I and *Pst*I to obtain 1.2 kb phytase gene, which was then ligated with *E. coli-Bacillus* shuttle vector, pJH27 (Jung, K.H. and Pack, M.Y., *Biotechnol. Lett.* 15:115-129, 1993) digested by the same restriction enzymes. *E. coli* MC1061 was transformed with the resulting plasmid, and about 1 x 10^6 transformants were obtained and subjected to DNA extraction. The extracted DNAs were introduced into *B. subtilis* DB431 to obtain a mutant library of phytase.

<Specific activity of mutant phytases prepared by the inventive random codon-based mutagenesis method>

Phytases having an enhanced specific activity were screened from the mutant library prepared in the above experiment, as follows. Colonies of the transformants in the mutant library were picked into 96 well plate containing 200 ㎕/well of a medium (0.2 % $(NH_4)_2SO_4$, 0.1 % sodium citrate, 0.02 % $MgSO_4$, 0.2 % Yeast extract, 0.5 % glucose, 1 mM $CaCl_2$, 50 ㎍/㎖ Tryptophan, 5 ㎍/㎖ kanamycin)

and cultured at 30℃ for 2 days. The culture medium was centrifuged at 4,200 rpm for 15 minutes to obtain a supernatant. The supernatant was diluted four times and 20 ㎕ of the resulting dilution was added to each well of a 96 well plate, 80 ㎕ of substrate (2 mM sodium phytate) was added to the well, and reaction was carried out at 37℃ for 2 hours. 100 ㎕ of staining reagent (4:1 mixture of 2.5 % ammonium molybdate and 2.5 % ferrous sulfate solution) was added to each well to allow color development. Absorbance of each well was determined using Microplate reader (Model 550, Bio RAD, USA) at 700 nm. Clones showing an absorbance higher than that of a control (wild-type phytase) were selected and cultured in a 5 ml test tube. The culture solution was taken at 20 and 24 hours after, respectively, and the activity of phytase was determined as above to select the clones showing a higher activity than the wild-type phytase.

The table given below shows the results of analyzing the specific activity of the mutant phytases selected as above and nucleotide sequences encoding the mutant phytases. The underlined nucleotide represents a mutation site where nucleotide substitution occurred.

<Table>

Phytase	Changes in amino acid and codon nucleotide sequences	Specific Activity (U/ml)
wild type	-	0.15
PT-RCM1	Lys^{27}(AA<u>G</u>), His^{28}(<u>CA</u>T) → Asn^{27}(AA<u>C</u>), Ser^{28}(<u>AG</u>T)	0.24
PT-RCM6	Lys^{27}(A<u>A</u>G) → Met^{27}(A<u>T</u>G)	0.23

PT-RCM12	Pro^{366}(CCG), Gln^{367}(CAG) → Pro^{366}(CCA), Glu^{367}(GAG)	0.21
PT-RCM17	Ala^{26}(GCC), Lys^{27}(AAG) → Ala^{26}(GCT), Glu^{27}(GAG)	0.22
PT-RCM29	Ile^{224}(ATC) → Thr^{224}(ACT)	0.28
PT-RCM42	His^{3}(CAT) → Ile^{3}(ATT)	0.19
PT-RCM46	Cys^{19}(TGC) → Phe^{19}(TTT)	0.24
PT-RCM52	Gly^{304}(GGG) → Gln^{304}(CAG)	0.50
PT-RCM54	Ala^{26}(GCC) → Tyr^{26}(TAT)	0.29
PT-RCM62	Gln^{25}(CAG), Ala^{26}(GCC) → His^{25}(CAC), His^{26}(CAC)	0.24

The result of the above experiment thus proves that the inventive random codon-based mutagenesis method is applicable to any DNA as a target and can be effectively used for the preparation of a protein having a desired property. This method is not implied by any prior art and is thus not obvious to the persons skilled in the art.

I hereby declare that all statements made herein of my own knowledge are true and all statements made on information and belief are believed to be true; and, further, that these statements made are punishable by fine or imprisonment, or both, under section 1001 of Title 18 of Unites States Code and that such willful false statements may jeopardize the validity of the application or any patents issuing thereon.

Further the deponent saith not.

Date: 13th day of February, 2006

Si Hyoung Lee

(Si-Hyoung LEE)

Exhibit

Exhibit A: Nucleotide and amino acid sequences of phytase

(Exhibit A)

Nucleotide and amino acid sequences of phytase

```
ATGAATCATTCAAAAACACTTTTGTTAACCGCGGCAGCCGGATTGATGCTCACATGCGGT    60
M  N  H  S  K  T  L  L  L  T  A  A  A  G  L  M  L  T  C  G      20
GCGGTTTCTTCCCAGGCCAAGCATAAGCTGTCTGATCCTTATCACTTTACCGTGAATGCG   120
A  V  S  S  Q  A  K  H  K  L  S  D  P  Y  H  F  T  V  N  A      40
GCGGCGGAAACGGAGCCGGTTGATACAGCCGGTGATGCAGCTGATGATCCTGCGATTTGG   180
A  A  E  T  E  P  V  D  T  A  G  D  A  A  D  D  P  A  I  W      60
CTGGACCCCAAGAATCCTCAGAACAGCAAATTGATCACAACCAATAAAAAATCAGGCTTA   240
L  D  P  K  N  P  Q  N  S  K  L  I  T  T  N  K  K  S  G  L      80
GTCGTGTACAGCCTAGAGGGAAAGACGCTTCATTCCTATCATACCGGGAAGCTGAACAAT   300
V  V  Y  S  L  E  G  K  T  L  H  S  Y  H  T  G  K  L  N  N     100
GTTGATATCCGCTATGATTTTCCGTTGAACGGAAAAAAAGTCGATATTGCGGCGGCATCC   360
V  D  I  R  Y  D  F  P  L  N  G  K  K  V  D  I  A  A  A  S     120
AATCGGTCTGAAGGAAAGAATACCATTGAGATTTACGCCATTGACGGGAAAAACAGCACA   420
N  R  S  E  G  K  N  T  I  E  I  Y  A  I  D  G  K  N  S  T     140
TTACAAAGCATTACAGATCCAGACCGCCCGATTGCATCAGCAATTGATGAAGTATACGGT   480
L  Q  S  I  T  D  P  D  R  P  I  A  S  A  I  D  E  V  Y  G     160
TTCAGCTTGTACCACAGTCAAAAAACAGGAAAATATTACGCGATGGTGACAGGGAAAGAA   540
F  S  L  Y  H  S  Q  K  T  G  K  Y  Y  A  M  V  T  G  K  E     180
GGCGAATTTGAACAATACGAATTAAATGCGGATAAAAATGGATACATATCCGGCAAAAAG   600
G  E  F  E  Q  Y  E  L  N  A  D  K  N  G  Y  I  S  G  K  K     200
GTAAGGGCGTTTAAAATGAATTCTCAGACAGAAGGGATGGCAGCAGACGATGAATACGGC   660
V  R  A  F  K  M  N  S  Q  T  E  G  M  A  A  D  D  E  Y  G     220
AGTCTTTATATCGCAGAAGAAGATGAGGCCATCTGGAAGTTCAGCGCTGAGCCGGACGGC   720
S  L  Y  I  A  E  E  D  E  A  I  W  K  F  S  A  E  P  D  G     240
GGCAGTAACGGAACGGTTATCGATCGTGCCGACGGCAGGCATTTAACCCCTGATATTGAA   780
G  S  N  G  T  V  I  D  R  A  D  G  R  H  L  T  P  D  I  E     260
GGACTGACGATTTACTACGCTGCTGACGGGAAAGGTTATCTGCTTGCATCAAGCCAGGGT   840
G  L  T  I  Y  Y  A  A  D  G  K  G  Y  L  L  A  S  S  Q  G     280
AACAGCAGCTACGCGATTTATGAAAGACAGGGACAGAACAAATATGTTGCGGACTTTCAG   900
N  S  S  Y  A  I  Y  E  R  Q  G  Q  N  K  Y  V  A  D  F  Q     300
ATAACAGACGGGCCTGAAACAGACGGCACAAGCGATACAGACGGAATTGACGTTCTGGGT   960
I  T  D  G  P  E  T  D  G  T  S  D  T  D  G  I  D  V  L  G     320
TTCGGGCTGGGGCCTGAATATCCGTTCGGCCTTTTTGTCGCACAGGATGGAGAAAATATA  1020
F  G  L  G  P  E  Y  P  F  G  L  F  V  A  Q  D  G  E  N  I     340
GATCACGGCCAAAAAGTGAATCAAAATTTTAAAATGGTGCCTTGGGAAAGAATCGCCGAT  1080
D  H  G  Q  K  V  N  Q  N  F  K  M  V  P  W  E  R  I  A  D     360
AAAATCGGCTTTCACCCGCAGGTCAATAAACAGGTTGACCCGAGAAAACTGACTGACAGA  1140
K  I  G  F  H  P  Q  V  N  K  Q  V  D  P  R  K  L  T  D  R     380
AGCGGAAAATAA                                                   1152
S  G  K  .                                                      383
```